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Transfected with DNA from Breast Cancer Cells

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This investigation was based on the hypothesis that weakly immunogenic, breast cancer-associated antigens, the products of mutant or dysregulated genes in the malignant cells, will be expressed in a highly immunogenic form by a semiallogeneic IL-2 secreting fibroblast transfected with DNA from breast cancer cells. To investigate this question, we transfected LM mouse fibroblasts (H2<sup>k</sup>) modified to secrete IL-2 with genomic DNA from breast adenocarcinoma that arose spontaneously in a C3H/He mouse (H2<sup>k</sup>). To increase their nonspecific immunogenic properties, the fibroblasts were also modified before transfection to express allogeneic MHC determinants (H-2K<sup>b</sup>). Afterward, the IL-2 secreting semiallogeneic cells were cotransfected with DNA from the spontaneous breast neoplasm, along with with a plasmid (pHyg) conferring resistance to hygromycin. Pooled colonies of hygromycin resistant cells were then tested in C3H/HeJ mice for their immunogenic properties against the growth of the breast neoplasm. The results indicated that the tumor-bearing mice immunized with the transfected cells survived significantly longer than mice in various control groups.

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## INTRODUCTION

These studies were undertaken to determine if immunization with semi-allogeneic fibroblasts modified to express IL-2 and transfected with genomic DNA from a breast tumor resulted in anti-breast tumor immunity. It had been previously determined in our laboratory that a similar vaccine constructed with melanoma genomic DNA was effective at generating anti-melanoma immunity (1).

We reasoned that genes specifying numerous, unidentified, weakly immunogenic TAAs would be expressed in a highly immunogenic form by the transfected cells, and that immunizations with the transfected cell would result in an immune response directed toward the breast cancer cell.

In this study, DNA was isolated from a spontaneously arising breast adenocarcinoma in a C3H/He mouse, spontaneous breast adenocarcinoma 1 (SB1), and was used to transfect LM cells, a mouse fibroblast cell line of C3H/He mouse origin. To increase their non-specific immunogenic properties and to ensure rejection, before transfection, the fibroblasts were modified to express allogeneic H-2K<sup>b</sup>-determinants, and to secrete IL-2. The results indicated that mice immunized with the transfected fibroblast developed immunity toward the breast cancer cells. The first appearance of tumor was delayed and the treated mice survived significantly longer than mice in various control groups, including mice injected with non DNA-transfected LM-IL-2K<sup>b</sup> fibroblasts.

These results raise the possibility that a fibroblast cell line that shares identity at one or more MHC class I alleles with the cancer patient may be readily modified to provide immunologic specificity for TAAs expressed by the patient's neoplasm.

**BODY:**

LM fibroblasts are of C3H/He mouse origin. They express H-2<sup>k</sup> class I determinants and share identity at the MHC with C3H/He mouse origin. To increase the fibroblasts' immunogenic properties, the cells were modified for IL-2 secretion and to express allogeneic (H-2K<sup>b</sup>) MHC-determinants (semi allogenic cells).

**Modification of LM mouse fibroblasts for IL-2-secretion.**

A replication-defective retroviral vector, pZipNeoSVIL-2, was used to modify LM cells (H-2<sup>k</sup>) for IL-2-secretion. The vector specified the gene for human IL-2, along with a gene (neo<sup>r</sup>) conferring resistance to the neomycin analog, G418. After selection in growth medium containing sufficient quantities of G418 to kill one hundred percent of non transfected cells, the surviving colonies were pooled and a biological assay for IL-2 was performed. The results (Table I) indicated that  $1 \times 10^6$  retrovirally transduced LM cells (LM-IL-2) formed approximately 100 units IL-2 in 48 hrs., as determined by the capacity of the culture supernatants to sustain the growth of IL-2-dependent CTLL-2 cells. LM-IL-2 cells modified for H-2K<sup>b</sup>-determinants (LM-IL-2K<sup>b</sup>; described, below), formed equivalent amounts of IL-2. The culture supernatants of LM cells, transduced with the IL-2-negative vector, pZipNeoSV(X), (LM-ZipNeo cells) failed to sustain the growth of CTLL-2 cells. Every third passage, the IL-2-secreting cells were placed in medium containing 400 µg/ml G418. Under these conditions, equivalent quantities of IL-2 were detected in the culture supernatants of each of the cell-types for more than 6 months of continuous culture (these data are not presented).

**Table I.** IL-2-secretion by genetically modified LM mouse fibroblasts transfected with pZipNeoSV-IL-2.

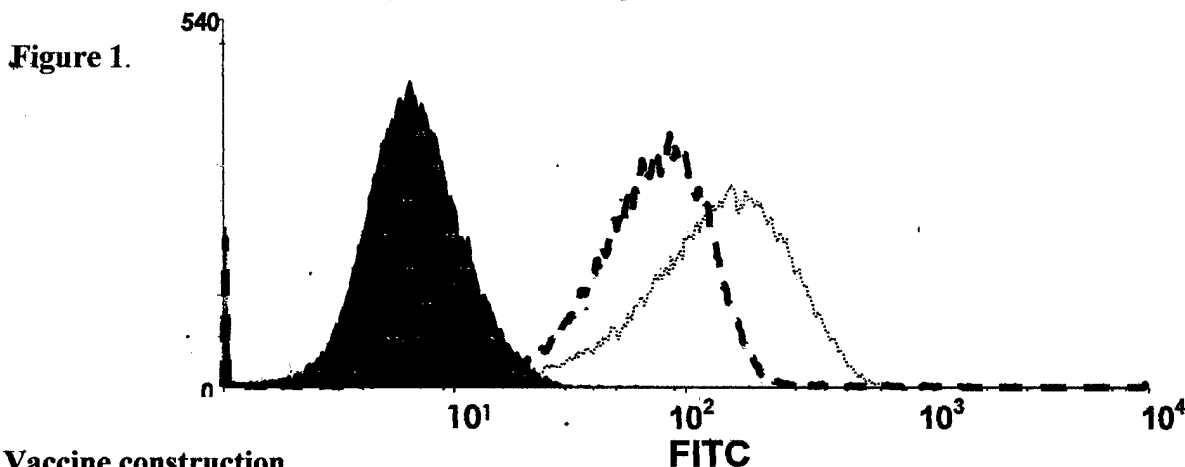
| Cell type             | IL-2<br>(units/ $10^6$ cells/48 hr) |
|-----------------------|-------------------------------------|
| LM-ZipNeo             | 0                                   |
| LM-IL-2               | 96                                  |
| LM-IL-2K <sup>b</sup> | 91                                  |

**Modification of LM-IL-2 cells for the expression of H-2K<sup>b</sup> class I determinants.**

LM-IL-2 cells were modified to express H-2K<sup>b</sup> determinants, by transfection with the plasmid pBR327H-2K<sup>b</sup> (2). A second plasmid, pBabePuro (3), conferring resistance to puromycin, was included for selection. (The ratio of pBR327H-2K<sup>b</sup> to pBabePuro used for transfection was 10:1). The transfected, puromycin-resistant cells were selected in growth medium containing sufficient quantities of puromycin (3µg/ml) to kill one hundred percent of non transfected LM-IL-2 cells. The surviving colonies were pooled and the cell number was expanded in vitro. The expression of H-2K<sup>b</sup>-determinants by the cells was measured by quantitative immunofluorescence, using FITC-labeled mAbs for mouse H-2K<sup>b</sup>. As controls, aliquots of the puromycin-resistant cell-suspension were incubated with FITC-labeled IgG2a isotype serum, or with FITC-labeled mAbs for H-2K<sup>k</sup> determinants. The results (Figure 1) indicated that puromycin-resistant LM-IL-2 cells co-transfected with pBR327H-2K<sup>b</sup> and pBabePuro stained

positively with H-2K<sup>b</sup> (thick line) and H-2K<sup>k</sup> (thin line) mAbs, but not with IgG2a isotype serum (shaded curve).

(LM cells are of C3H mouse origin). The expression of H-2K<sup>b</sup>-determinants appeared to be a stable property of the cells. They stained with equivalent intensity with H-2K<sup>b</sup> mAbs after six months of continuous culture (these data are not presented).



#### **Vaccine construction**

Shared, unfractionated DNA isolated (Qiagen, Chatsworth, CA) from a spontaneous mammary adenocarcinoma (SB1) taken directly from a C3H/HeJ mouse, or from EO771 cells taken from a C57BL/6 mouse, was used to transfect LM-IL-2K<sup>b</sup> cells. The method described by Wigler et al. (4) was applied, as modified. Briefly, high molecular weight DNA from each cell type was sheared by three passages through a 25 gauge needle. Afterward, 100 µg of the sheared DNA was mixed with 10 µg pHyg (from L. Lau, University of Illinois, Chicago, IL), a plasmid that encoded the *E. Coli* enzyme hygromycin B phosphotransferase (5), conferring resistance to Hygromycin B. The sheared DNA and pHyg were then mixed with Lipofectin, according to the manufacturer's instructions (Gibco BRL). The DNA/Lipofectin mixture was added to a population of  $1 \times 10^7$  LM-IL-2K<sup>b</sup> cells that had been divided into ten 100 mm plastic cell culture plates 24 hrs. previously. Eighteen hrs. after addition of the DNA/Lipofectin mixture to the cells, the growth medium was replaced with fresh growth medium. For use as a control, DNA from the tumor cells was omitted and 1 µg of pHyg alone, mixed with Lipofectin, was added to an equivalent number of LM-IL-2K<sup>b</sup> cells. In each instance, the cells were maintained for 14 days in growth medium containing 600 µg/ml hygromycin B (Boehringer Mannheim, Indianapolis, IN). One hundred percent of LM-IL-2K<sup>b</sup> transfected with tumor-DNA alone maintained in the hygromycin-growth medium died within this period. The surviving colonies (at least  $2.5 \times 10^4$ ) of LM-IL-2K<sup>b</sup> cells transfected with pHyg and DNA from the tumor cells, or with pHyg alone (LM-IL-2K<sup>b</sup> cells), were pooled and used in the experiments.

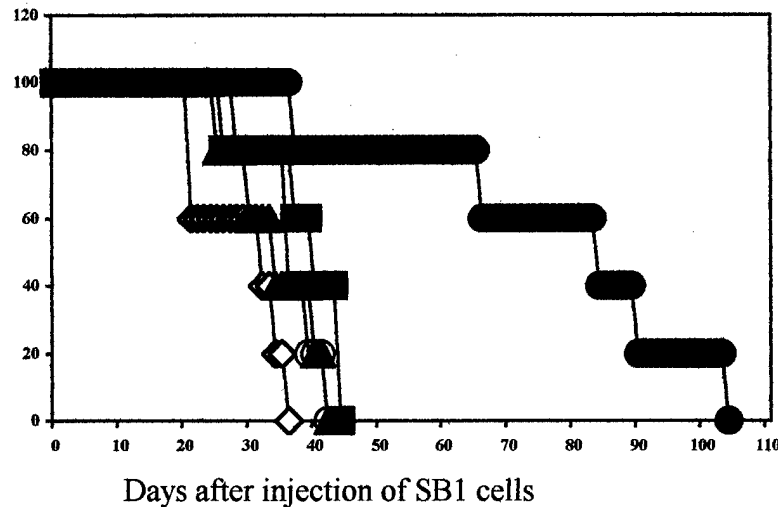
#### **Survival of C3H mice injected with SB1 spontaneous mammary adenocarcinoma cells and LM-IL-2K<sup>b</sup> cells transfected with DNA from SB1 cells.**

C3H/HeJ mice exhibit no apparent resistance to the growth of SB1 mammary adenocarcinoma cells. One hundred percent of naive mice injected intra breast fat pad with  $1 \times 10^6$  SB1 cells die from progressive tumor growth.

The potential immunotherapeutic properties of LM-IL-2K<sup>b</sup>/SB1 cells were determined by injecting C3H/HeJ mice into the fat pad of the breast with a mixture of  $1 \times 10^6$  SB1 cells and  $2 \times 10^6$  LM-IL-2K<sup>b</sup>/SB1 cells (●). At the same time, the mice received a second injection i.p. of  $2 \times$

$10^6$  LM-IL-2K<sup>b</sup>/SB1 cells alone. As controls, other naive C3H/HeJ mice were injected according to the same protocol with equivalent numbers SB1 cells and LM-IL-2 cells (■), with SB1 cells and LM-IL-2K<sup>b</sup> (▲) cells, with SB1 cells and LM-IL-2/SB1 (○) cells or with SB1 cells alone (◇). The mice in each treatment group were injected twice more, at weekly intervals, with the same number of LM-IL-2K<sup>b</sup>/SB1, LM-IL-2 cells, LM-IL-2K<sup>b</sup> or LM-IL-2/SB1 cells, but without additional SB1 cells.

**Figure 2.**



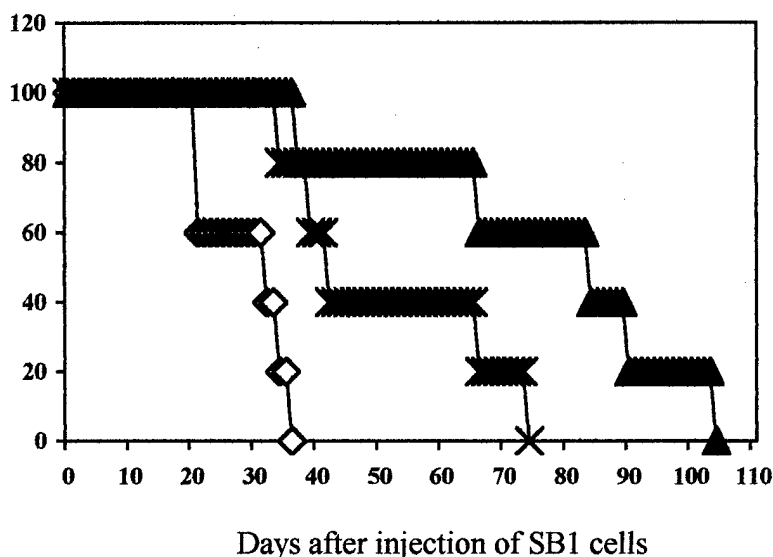
As indicated (Figure 2), the first appearance of tumor, rate of tumor growth and survival of mice in each group was approximately the same as that of mice injected with SB1 cells alone. The greatest immunotherapeutic benefit was in the group of mice injected with the mixture of SB1 cells and LM-IL-2K<sup>b</sup>/SB1 cells.

#### **The effect of tumor DNA on the specificity of the immune response**

To determine if LM-IL-2 K<sup>b</sup> cells transfected with the DNA from a tumor (EO771), which has a histological morphology similar to SB1 yet is from a different host strain (LM-IL-2K<sup>b</sup>/EO771) could effect the immune response against the growth of SB1 cells, naive C3H/HeJ mice were injected with a mixture of SB1 cells and LM-IL-2K<sup>b</sup>/EO771 cells. C3H/HeJ mice (7 per group) were injected s.c. with a mixture of  $1 \times 10^6$  SB1 cells and  $2 \times 10^6$  LM-IL-2K<sup>b</sup>/SB1 cells (▲). At the same time, the mice received a second injection i.p. of  $2 \times 10^6$  LM-IL-2K<sup>b</sup>/SB1 cells alone. As controls, other naive C3H/HeJ mice were injected according to the same protocol with equivalent numbers SB1 cells and LM-IL-2K<sup>b</sup>/EO771 cells (×), or with SB1 cells alone (◇). The mice in each treatment group were injected twice more, at weekly intervals, with the same number of LM-IL-2K<sup>b</sup>/SB1, LM-IL-2K<sup>b</sup>/EO771, or with media alone, but without additional SB1 cells.



**Figure 3.**



As indicated (Figure 3), although mice injected with the mixture of SB1 cells and LM-IL-2K<sup>b</sup>/EO771 cells survived longer than mice injected with SB1 cells alone, they died in significantly ( $P < .01$ ) shorter intervals than mice injected with SB1 cells and LM-IL-2K<sup>b</sup> cells transfected with DNA from SB1 cells. The reverse experiment in which C57BL/6 mice injected with EO771 cells were treated with LM-IL-2K<sup>b</sup>/EO771 or LM-IL-2K<sup>b</sup>/SB1 resulted in similar findings (data not shown).

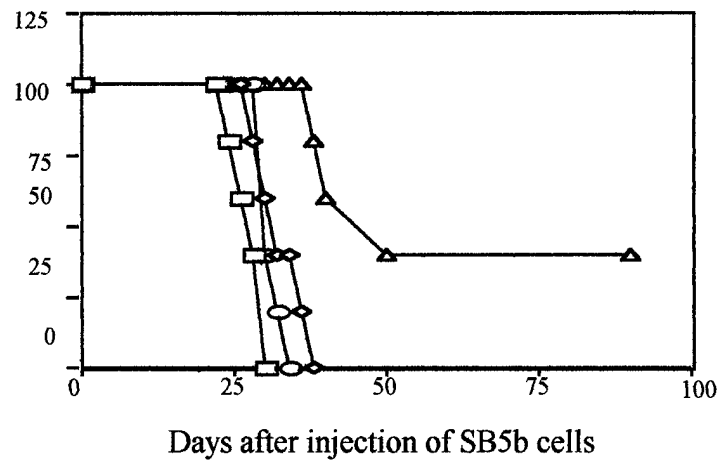
Although there was some cross reaction in the response against both vaccines a more potent response was elicited by the vaccine transfected with the DNA from the tumor being treated.

#### **Involvement of CD8<sup>+</sup> T cells in the anti-breast tumor immune response**

Since CD8<sup>+</sup> T cells are the primary effector population responsible for specific, cytolytic anti-tumor immunity, experiments were performed to determine if CD8<sup>+</sup> T cells were required for the effectiveness of this type of therapy. For this purpose I depleted CD8<sup>+</sup> T cell subsets in vivo. This was accomplished by injecting mice with rat mAb anti-mCD8 (ATCC hybridoma 2.43). C3H mice received three s.c. injections at weekly intervals of a vaccine prepared by transfection of modified LM fibroblasts with DNA from an adenocarcinoma of the breast that arose spontaneously in a C3H mouse (SB-5b). Each injection consisted of  $5 \times 10^6$  cells. One week after the last immunization, the mice were injected with  $1 \times 10^4$  breast cancer cells. CD8 antibodies were injected at 1mg/injection/mouse on days -2 and 0, relative to tumor injection, and every 5 days thereafter throughout the duration of the experiment.

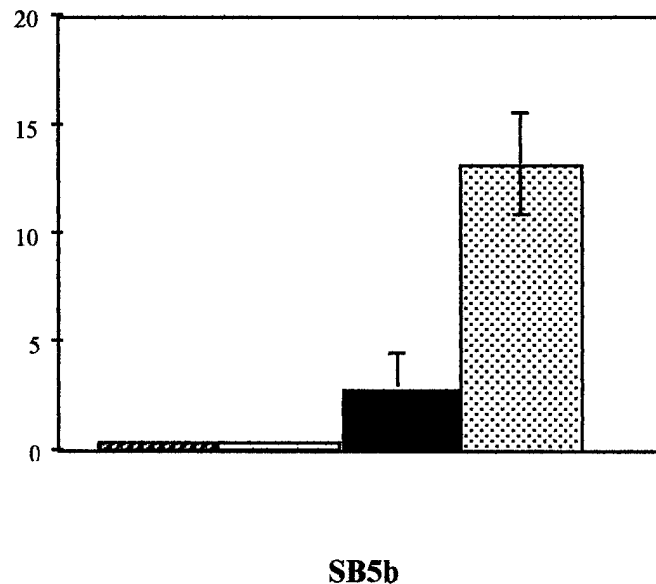
As indicated (Figure 4), the first appearance of tumor and survival of mice immunized with LM-IL-2K<sup>b</sup>/SB5b depleted of CD8<sup>+</sup> cells (○) was not significantly different from the first appearance of tumor and survival of mice injected with SB5b cells alone (□) or non transfected LM-IL-2K<sup>b</sup> cells (◇). Immunization of mice with LM-IL-2K<sup>b</sup>/SB5b vaccine (△) prolonged their lives and 40% of the mice remained tumor free 90 days after challenge with tumor. Thus, depletion of CD8<sup>+</sup> T cells affected the animals capacity to resist the growth of the breast cancer cells in mice immunized with DNA-transfected cells.

**Figure 4**



Further experiments were performed to determine if cytotoxic activity toward breast cancer cells (SB5b) was mediated by CD8<sup>+</sup> T cells. Spleen cells were isolated from mice immunized with LM-IL-2K<sup>b</sup>/SB5b (dotted bars), mice immunized with LM-IL-2K<sup>b</sup>/SB5b and depleted of CD8 cells (closed bars), mice immunized with LM-IL-2K<sup>b</sup> cells (open bars) or naive mice (hatched bars). All mice received three injections at weekly intervals of  $5 \times 10^6$  cells. <sup>51</sup>Cr release assay was performed one week after the last immunization. Spleen cells were incubated with Cr-labeled SB5b cells at a 100:1 ratio.

**Figure 5**



The results (Figure 5) indicate that the highest responses toward the breast cancer cells were in the mice immunized with LM-IL-2K<sup>b</sup>/SB5b cells. Cytotoxicity was mediated by CD8<sup>+</sup> cells, since specific lysis of SB5b tumor was significantly inhibited when spleen cells obtained from mice immunized with LM-IL-2K<sup>b</sup>/SB5b and depleted of CD8 cells were reacted with SB5b tumor. Mice immunized with non DNA transfected LM fibroblasts modified to secrete IL-2 and to express allogeneic (H-2Kb) determinants also failed to exhibit cytotoxicity toward the breast cancer cells.

## KEY RESEARCH ACCOMPLISHMENTS

- a) Isolated DNA from eight spontaneous breast cancer cells arising in C3H/HeJ mice
- b) Modified LM fibroblasts to form IL-2. Confirmed IL-2 secretion
- c) Modified LM-IL-2 cells to express H-2K<sup>b</sup> determinants
- d) Transfected LM-IL-2K<sup>b</sup> cells with DNA from different breast neoplasms (SB1, SB5b) arising in individual C3H/HeJ mice
- e) Treated mice with SB1 breast cancer with fibroblasts transfected with DNA from SB1 cells. Measured tumor growth in these mice and determined that the treatment resulted in anti-breast cancer immunity.
- f) Treated mice with SB1 breast cancer with fibroblasts transfected with DNA from breast cancer arising in a different strain of mice (EO771). The treatment resulted in prolongation of survival of mice with SB1 tumors indicating cross reaction of antigens between the two breast tumors.
- g) Determined that CD8<sup>+</sup> T cells were required for the anti-tumor response in mice immunized with SB5b transfected LM-IL-2K<sup>b</sup> cells.

## **REPORTABLE OUTCOMES**

Manuscript in progress.

## CONCLUSIONS:

The data presented here indicate that semi-allogeneic fibroblasts modified to express IL-2 and breast tumor antigens, can activate a potent, specific anti-breast immune response. There is evidence for the development of cytotoxicity mediated by CD8<sup>+</sup> T cells.

Like other neoplasms, breast cancer cells form TAAs, several of which have been identified (6,7). However, antigens associated with the proliferating malignant cells are insufficiently immunogenic to generate an effective immune response. Proliferating breast cancer cells fail to elicit anti tumor responses that can control tumor cell-growth.

Here, I tested a unique approach toward the introduction of tumor antigens into a host. I combined two classic findings. The first is that transfection of high molecular weight genomic DNA from one cell-type can alter both the genotype and the phenotypic characteristics of the cells that take-up the exogenous DNA. (8-11)

The second finding, is that the genotype of tumor cells differs from normal, nonmalignant cells of the tumor-bearing host. I hypothesized that undefined, altered genes specifying TAAs would be expressed in a highly immunogenic form by a subpopulation of cells transfected with DNA from the breast cancer cells, and that the number of such cells would be sufficient to generate the anti tumor immune response. Using C3H/HeJ mice and SB1 (spontaneous mammary adenocarcinoma) as our model, we utilized LM-IL-2 cells (syngeneic in this model) and transfected them with genomic DNA from SB1 tumor and then compared the effect of this cell construct with the effect of LM-IL-2K<sup>b</sup> cells transfected with SB1 tumor DNA. The results (Figure 2) indicate that tumor growth was delayed in the mice injected with LM-IL-2K<sup>b</sup>/SB1 and that LM-IL-2/SB1 had no effect on the survival or the growth kinetics in C3H/HeJ mice. The failure of transfected cells that expressed syngeneic MHC-determinants alone to induce an anti breast cancer response reaffirmed the important role of allogeneic determinants in the cells' overall immunogenic properties.

These results concur with results from Toes et al. (12) who found that a completely allogeneic tumor could elicit a strong immune response against a specific syngeneic MHC restricted peptide.

Work by Kim et al. (13) has indicated that tumor immunity elicited by LM-IL-2/B16 is specific to the tumor from which the DNA is derived yet in these experiments the tumors which are compared are tumors that stem from different cell types and are of completely different histological morphology. Using a more stringent model to determine the specificity of the anti tumor immune response elicited by the cell construct we utilized E0771 tumor which is a mouse mammary adenocarcinoma that has a similar morphology to the SB1 mammary adenocarcinoma cells. We compared the growth of tumor and the time of survival of mice injected with SB1 and LM-IL-2K<sup>b</sup>/SB1, LM-IL-2K<sup>b</sup>/E0771 or media (Figure 3). We found that while LM-IL-2K<sup>b</sup>/SB1 prolonged the survival of mice, with SB1 tumor, longer than mice injected with LM-IL-2K<sup>b</sup>/E0771 there was still significant prolongation of survival in the mice injected with LM-IL-2K<sup>b</sup>/E0771. These results indicate the existence of shared tumor associated antigens or possibly the cross reaction of some antigens between the two breast tumors of similar morphology.

The data reported here raise the possibility that an antigen-presenting cell line that shares identity at one or more MHC class I alleles with the cancer patient may be readily modified to provide immunologic specificity for TAAs expressed by the patient's neoplasm. Transfection of the cell line with DNA from the patient's neoplasm may provide a practical alternative to the modification of autologous malignant cells for the purposes of tumor immunotherapy.

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